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Published in:
Journal of Bacteriology

IMPORTANT NOTE: You are advised to consult the publisher's version (publisher's PDF) if you wish to cite from it. Please check the document version below.

Document Version
Publisher's PDF, also known as Version of record

Publication date:
1996

[Link to publication in University of Groningen/UMCG research database](#)

Citation for published version (APA):

Franke, C. M., Leenhouts, K. J., Haandrikman, A. J., Kok, J., Venema, G., & Venema, K. (1996). Topology of LcnD, a protein implicated in the transport of bacteriocins from *Lactococcus lactis*. *Journal of Bacteriology*, 178(6), 1766-1769.

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Topology of LcnD, a Protein Implicated in the Transport of Bacteriocins from *Lactococcus lactis*

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Received 21 July 1995/Accepted 8 January 1996

Four in-frame translational fusions to both the reporter proteins β -galactosidase and alkaline phosphatase support a topological model of LcnD, a protein implicated in the transport of several bacteriocins from *Lactococcus lactis*, in which the N-terminal part is located intracellularly and one transmembrane helix spans the cytoplasmic membrane.

Lactococcins are bacteriocins produced by *Lactococcus lactis* (15, 17, 18). They belong to the small heat-stable, non-lanthionine-containing membrane-active peptides characterized by a Gly-Gly⁻¹-X⁺¹ (X is any amino acid) processing site in the precursor. The proteins LcnC and LcnD are essential for extracellular activity of these bacteriocins, and a role in transport and/or maturation has been suggested (15). LcnC, a member of the ABC transporter family, is a putative transmembrane protein with several transmembrane sequences (TMS). LcnD does not show significant amino acid sequence similarity with other proteins. Computer predictions indicate that LcnD contains one TMS between amino acid residues 21 and 44. To test this topological model, we constructed four in-frame translational fusions with β -galactosidase (LacZ) (8) and alkaline phosphatase (PhoA) (1, 8). The fusion proteins were encoded by pWV01 derivatives (20) under control of the strong lactococcal P32 promoter (21), and activities could thus be tested in both *Escherichia coli* and *L. lactis*.

By standard PCR techniques (7), unique restriction enzyme sites were introduced in *lcnD* to generate fusions with *lacZ* or *phoA*. In the topological model of LcnD (Fig. 1), the amino acids shown as black circles correspond to residues to which either β -galactosidase or alkaline phosphatase was fused. The activities of β -galactosidase and alkaline phosphatase fusions were measured (1, 10) in triplicate in cell-free extracts (CFE) and plate assays (Table 1).

LacZ fusions. In both *E. coli* and *L. lactis*, activities were highest when LacZ was fused to the N-terminal moiety of LcnD, whereas they were negligible when LacZ was fused to the C terminus (Table 1). Fusion of LacZ to LcnD at position 30 in the putative TMS led to a chimeric protein with an activity 50 times lower than that of a protein in which LacZ was fused upstream of the putative TMS (at position 21). This is the case in both organisms and supports the presence of a transmembrane segment at this position. All fusions of LcnD with LacZ show comparable relative activities in *E. coli* and *L. lactis*, which suggests similar topologies in both organisms.

PhoA fusions. Because of instability problems with the *lcnD-phoA* constructs, we placed these behind the inducible *E. coli*

lac promoter (23). Plasmids expressing the LcnD-PhoA chimeras were unstable in *L. lactis*. Even integration of the constructs into the lactococcal chromosome to ensure a low copy number led to deletion formation, as judged by Southern hybridization, and no fusion products were identifiable in Western immunoblots (data not shown). When PhoA was fused in or upstream of the putative TMS, it showed no significant activity in *E. coli*, whereas the C-terminal fusion activities were clearly higher than background activity (Table 1). In all cases, the PhoA fusion proteins showed properties complementary to the LacZ chimeras. The fact that *E. coli* CC118 colonies expressing the LcnD-PhoA fusion in the putative TMS are slightly blue in the plate assay might indicate the presence of the TMS at this position.

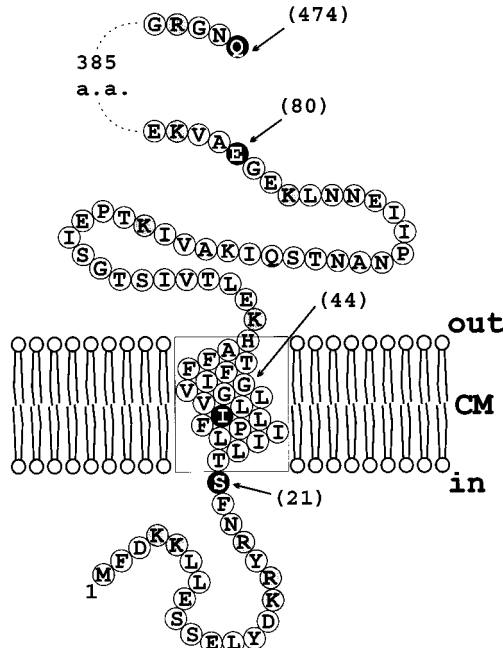
Western blot analysis. Synthesis of fusion proteins was verified by Western hybridizations with Western-Light (Tropix, Bedford, Mass.) and nitroblue tetrazolium-5-bromo-4-chloro-3-indolylphosphate toluidinium (NBT/BCIP; Promega, Madison, Wis.) and anti-LacZ and anti-PhoA antibodies. Products of the expected sizes could indeed be detected, but breakdown products were also present (Fig. 2). A positive band in the CFE of *E. coli* WK6 corresponded to the size of β -galactosidase Δ M15 produced by this strain (Fig. 2A and B, lanes 1). *E. coli* WK6 containing pMG57, a plasmid carrying the entire *E. coli lacZ* gene without its ribosome-binding site (RBS) (19), showed an additional band similar in size to that of partially purified β -galactosidase. All strains carrying *lcnD::phoA* fusions were induced with IPTG (isopropylthiogalactopyranoside), and similar amounts of protein were produced (Fig. 2C).

Electron microscopy. Cells from overnight cultures of *E. coli* expressing the LcnD-LacZ fusions were fixed at 0°C in 2.5% formaldehyde–0.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2) and dehydrated in a graded ethanol series. Fixed cells were then embedded in Unicryl resin. Ultrathin sections were labeled with rabbit anti-LacZ antibodies, which were subsequently detected with immunogold-labeled goat anti-rabbit polyclonal antibodies. Electron microscopic images were obtained on a Philips CM10 transmission electron microscope (Philips, Eindhoven, The Netherlands). In *E. coli* WK6, hardly any label was detected (Fig. 3). In cells containing pMG57, little label is found, whereas cells expressing LcnD fused to LacZ at residue 21 or at position 30 show a considerable quantity of label in the cytoplasm, indicative of the presence of LacZ antigen in this cell compartment. In cells expressing LcnD fused to LacZ at position 80 or 474, label was found almost exclusively near the cytoplasmic membrane. Ap-

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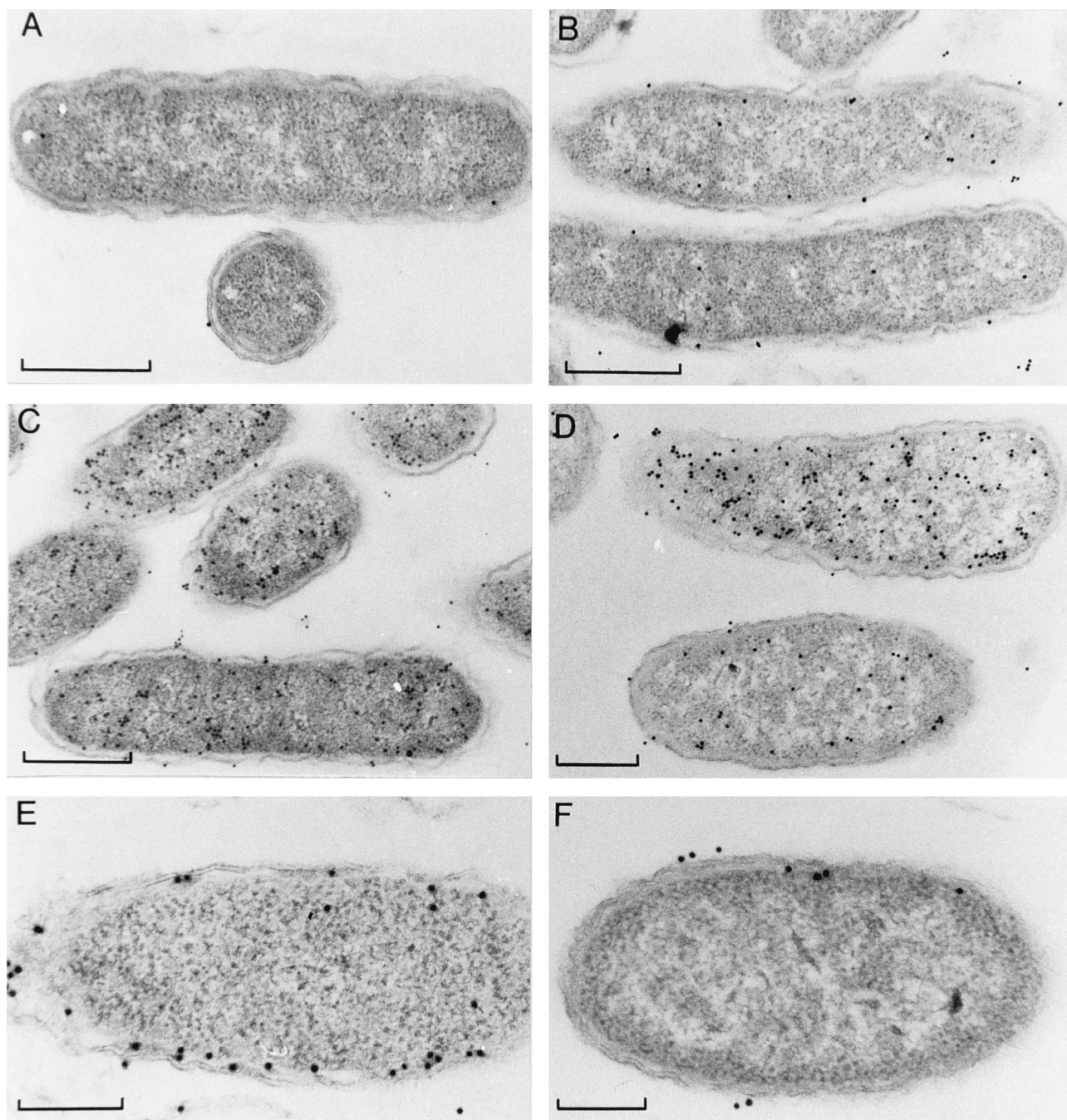


FIG. 3. Electron microscopy images of immunogold-labeled ultrathin sections of fixed overnight cultures of *E. coli* WK6 carrying (A) no plasmid, (B) pMG57, and (C to F) plasmids encoding β -galactosidase fused to LcnD at positions 21, 44, 80, and 474 (Fig. 1), respectively. The first antibody was rabbit anti- β -galactosidase. The second was goat anti-rabbit antibody labeled with 15-nm gold particles. Bars, 0.5 μ m.

the outside of the cytoplasmic membrane are susceptible to protease activity, conceivably because of incorrect folding. In *L. lactis*, such products were hardly detectable. The relative activities of all LcnD-LacZ fusion proteins in exponentially growing cells and overnight cultures were comparable except for the C-terminal fusion protein, the activity of which was significantly higher in the overnight culture. This result suggests more pronounced proteolytic degradation in overnight cultures, as was indeed shown by Western hybridization (Fig.

2B). It supports our notion of proteolytic breakdown leading to background LacZ activity.

From computer predictions, topological models have been developed for proteins forming dedicated transport systems for lactococcins (LcnC and LcnD) (15, 17), pediocin (PedC and PedD) (9), and hemolysin (HlyB and HlyD) (3). Each transport system has one member of the family of ABC transporters (LcnC, PedD, and HlyB, respectively). The accessory proteins HlyD (13) and PedC (2a) have a membrane topology similar to

that of LcnD, and although they do not have amino acid sequence similarities, they seem to belong to a group of proteins with similar functions. All three are important for the production of active extracellular bacteriocin (LcnD and PedC) and hemolysin (HlyD). Their conserved genetic organization and topological structure suggest an important function in secretion of their respective allocrites.

We thank Klaas Sjollem for electron microscopy and Henk Mulder for photography.

K. Venema was supported by the EC BRIDGE T-project on LAB. J. Kok holds a fellowship from the Royal Netherlands Academy of Arts and Sciences (KNAW).

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